Abstract

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Sleep apnea affects approximately a quarter of all Americans. The effects of sleep apnea involve a decrease in testosterone levels, along with an increased rate of hypertension, stroke, and coronary heart disease. Chronic Intermittent Hypoxia (CIH) has also been shown to increase aging and negatively affect cognitive function, as seen with increased oxidative stress within the Entorhinal Cortex. However, it is unknown if Testosterone Replacement Therapy (TRT) can ameliorate the impact of CIH on the entorhinal cortex, which could have significant therapeutic impacts on sleep apnea related cognitive impairment. We hypothesized that TRT would mitigate protein levels of oxidative stress and inflammation, as measured by COX2, GFAP, and Calpain enzymatic activity. Banked brain tissue from young (3 month) F344/BN F1 hybrid male rats were used. Rats were separated into three treatment groups: gonadally intact, gonadectomized (GDX), and GDX with TRT. Then rats were either exposed to room air (normoxic conditions) or CIH for 8 days.

Our results showed that neither TRT nor CIH impacted inflammation and oxidative stress related proteins in the entorhinal cortex. However, rats that were exposed to isoflurane anesthesia (GDX and GDX+TRT) showed neuroinflammation. Cognitive impairment has been associated with isoflurane related surgeries. However, the mechanism is unknown, though based on the data inflammation may be involved in isoflurane induced cognitive impairment in surgical patients.

Testosterone Replacement Therapy: Role in Modulating Oxidative Stress within the Entorhinal Cortex

PRACTICUM REPORT

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CHPATER I. BACKGROUND AND LITERATURE

Sleep Apnea Incidence

Often undiagnosed, sleep apnea is characterized by episodes of upper airway collapse. This leads to hypoxia and interruption of sleep. Sleep apnea occurs among 24% of men and 9% of women, with higher rates among obese individuals. This rate increases to 70% in older men and 56% in older women [1]. Men tend to have a higher Apnea-Hypopnea Index (AHI), indicating more severe sleep apnea. This leads to greater disruptions in sleep cycles and ultimately a greater cost to health and society [2]. Sleep apnea is estimated to cost around $150 billion annually. This includes costs from loss of productivity, motor vehicle accidents, and workplace accidents. Within this estimate, $30 billion comes from undiagnosed sleep apnea leading to increased health care utilization [3]. Among these healthcare costs includes the potential for sleep apnea to damage the entorhinal cortex.

Sleep Apnea and the Entorhinal Cortex

Sleep apnea has been shown to increase levels of oxidative stress and hypoxia in individuals. These results have been shown as underlying factors in the progression of Alzheimer's disease. Though the relationship is potentially bidirectional, there are indications of addressing sleep apnea as a potential for decreasing the risk of Alzheimer's disease [4]. Sleep apnea has been shown to cause an increase in oxidative stress levels within the entorhinal cortex. This region of the brain is a major pathway in the development of Alzheimer's disease [5]. Another major area of concern for damage to the entorhinal cortex within rats is damage to cognitive functions.

Chronic intermittent hypoxia has been shown to cause an impairment in the Morris Water Maze trial in male rats, indicating a decline in spatial memory [6].

Neuroprotective Effects of Androgens

Neurodegeneration has been shown to increase with aging in individuals. Within mouse models, lack of testosterone has shown an increase in oxidative stress [7]. Testosterone has been shown to combat the effects of this problem through recruitment of nearby neurons following neuronal death [8, 9]. This increase in neurogenesis in adults has been potentially linked to indirect pathways involving brain-derived neurotrophic factor and glucocorticoids. Though the complete pathway of this neurogenesis is not known, studies have shown that the neuroprotective effects of testosterone to oxidative stress via catalase activity of 3-nitro-L-tyrosine [10, 11]. The increased catalase activity may deal with cellular antioxidant defense system.

Testosterone Replacement Therapy (TRT)

Though the usage of TRT in young men is associated with no adverse effects, there is not a consensus in the field about the use of TRT in older male populations. Currently, TRT is only approved by the FDA for treatment of low testosterone due to hypogonadism and not for patients with age-related low testosterone [12].

Literature regarding TRT is equivocal. Though research shows the neuroprotective effects of TRT against oxidative stress, there is still concern around the other effects of TRT on the body, specifically on cardiovascular health [13-15]. Our published data shows that chronic intermittent hypoxia (CIH) decreased testosterone levels and increased oxidative stress (**Figure 1**) in male rats. Exposure to TRT blocked CIH induced oxidative stress in male rats [16], indicating that TRT was

neuroprotective. However, the neural mechanisms of mediating TRT inhibition of oxidative stress in young male rats in unknown.

Chronic Intermittent Hypoxia (CIH) Model

There are major areas of concern in terms of the effects of sleep apnea on the body. Sleep apnea induces hypoxia due to the obstruction of the airway. To examine the pathophysiology of sleep apnea, we use an animal model called chronic intermittent hypoxia (CIH).

Rats do not experience sleep apnea. However, we can model the hypoxia associated with obstructive sleep apnea by manipulating the oxygen levels that the rats breathe when they are asleep. Within rats, CIH can be induced by exposing rats to 8-minute cycles of 5 minutes of 10% O_2 and 3 minutes of 21% O_2 from 1700 to 0500h for 8 days. This allows for the effects of sleep apnea to be studied within animal models [16].

Oxidative Stress, Inflammation, and Cognitive Function

Oxidative stress has a bidirectional relationship with inflammation. Both oxidative stress and inflammation can negatively impact the brain, such as the entorhinal cortex that is involved in cognitive function [6]. Both oxidative stress and Inflammation have been shown to increase rates of neurodegeneration in individuals [17, 18]. Among older populations, this serves to be a greater medical issue as it can lead to a higher incidence rate of Alzheimer's disease and a subsequent decrease in cognitive function. A buildup of extracellular amyloid plaques and amyloid β-peptide resulting from oxidative stress within the entorhinal cortex is particularly problematic, as this can cause synapse loss in a region is heavily implicated in the onset of decreased cognitive function. [19, 20].

Cellular markers of oxidative stress and inflammation include Calpain protein cleavage, Glial Fibrillary Acidic Protein (GFAP), and Cyclooxygenase-2 (COX2). Calpain, an oxidative stress associated enzyme, can be used to measure oxidative stress activity within cells by examining its cleavage of other proteins, such as the cytoskeletal protein Spectrin. Calpain serves as a Ca^{2+}-dependent cysteine protease in neurons [21]. GFAP is expressed within activated astrocyte cells, usually after an insult occurs. GFAP serves to cause neurogenesis within these cells and can therefore be seen to be highly expressed when inflammation occurs within a region [22, 23]. COX2 is expressed in neurons during inflammation by proinflammatory cytokines and serves to release prostanoids, producing localized pain hypersensitivity [24, 25].

SIGNIFICANCE

Sleep apnea affects approximately a quarter of all Americans, with a higher incidence rate among men [6]. The effects of sleep apnea involve a decrease in testosterone levels, along with an increased rate of hypertension, stroke, and coronary heart disease [26]. Within young rats, CIH has been shown to increase signs of aging, as evidenced by elevated oxidative stress [16]. Cognitive function has also been shown to be affected by sleep apnea induced CIH. These include decreases in attention and memory [27]. Elevated oxidative stress can impact the entorhinal cortex region, a brain region associated with cognitive function, resulting in cellular oxidative stress and inflammation [28]. However, it is unknown if TRT can ameliorate the impact of CIH on the entorhinal cortex, which could have significant therapeutic impacts on sleep apnea related cognitive impairment.

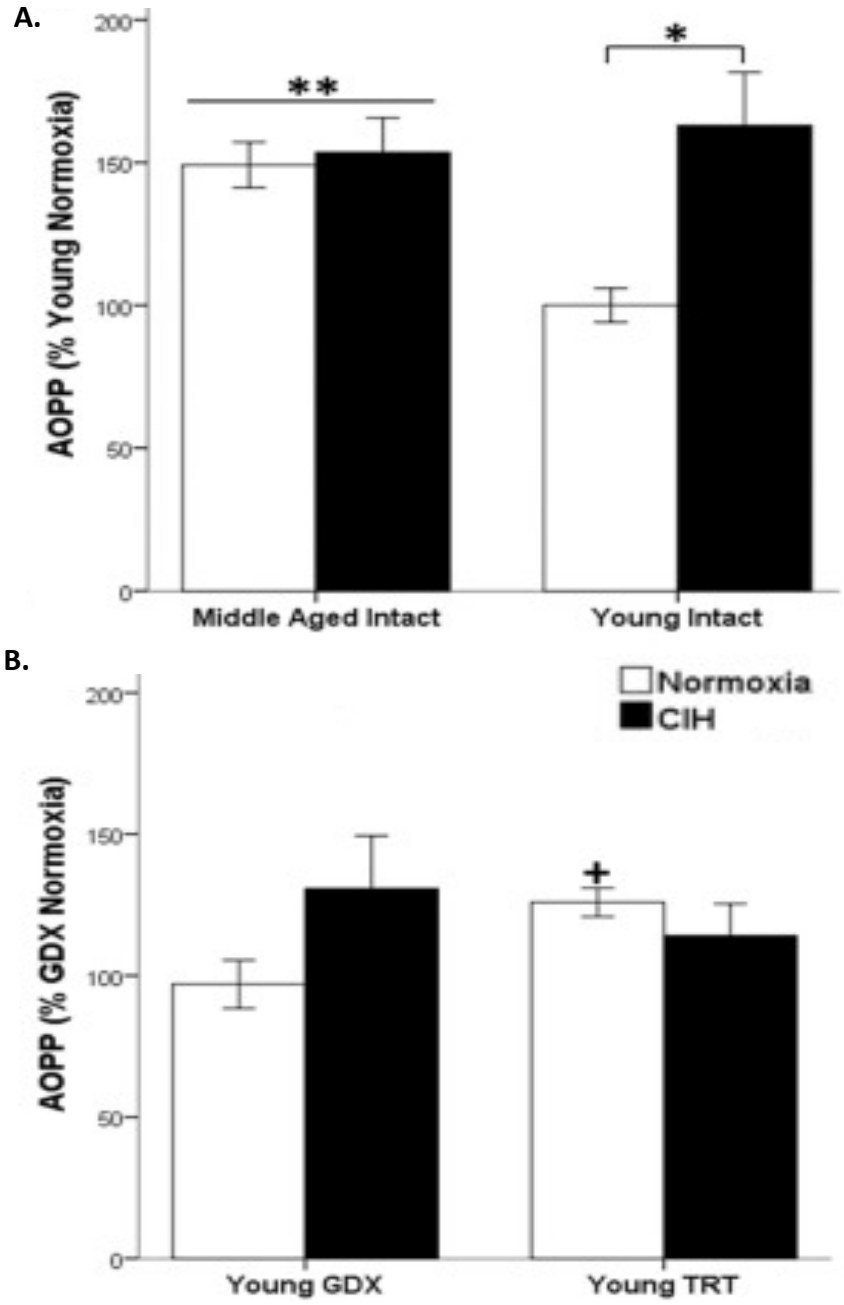

Figure 1. CIH increased oxidative stress (AOPP) in young gonadally intact male rats (A). CIH did not increase oxidative stress in GDX or TRT rats. TRT did increase oxidative stress in control rats, indicating testosterone can act as an oxidative stressor (B). * vs CIH, ** vs young normoxia, + vs GDX normoxia. Modified from Wilson 2018.

CHAPTER II: RESEARCH PROJECT

SPECIFIC AIMS

Specific Aim 1: Analyze the role of chronic intermittent hypoxia on oxidative stress generation within the entorhinal cortex and the impact of testosterone replacement therapy on oxidative stress.

Specific Aim 2: Analyze the role of chronic intermittent hypoxia on inflammation within the entorhinal cortex and the impact of testosterone replacement therapy on inflammation.

We hypothesize that CIH will increase oxidative stress and inflammation related proteins in the entorhinal cortex of gonadally intact rats, while neither GDX nor TRT rats will show increased expression of oxidative stress or inflammation related markers in response to CIH in the same region.

MATERIALS AND METHODS

Animal Tissue

Brain tissue was obtained from banked tissue from a prior study examining circulating oxidative stress markers and behavior in young (3 month) F344/BN F1 hybrid male rats from the National Institute of Aging (through Envigo, Indianapolis, IN). Sexually naïve rats were used to remove potential confounding factors that arise from increased testosterone levels from sexual experience [16]. Rats were double-housed in temperature-controlled room (23°C), being habituated for a week with a 12:12 light/dark cycle with lights off at 0900h. Rats were then separated into three treatment groups: gonadally intact, gonadectomized (GDX), and GDX with testosterone replacement therapy (TRT). Surgery was performed under isoflurane (2-3%) anesthesia, and no sham surgery was performed on gonadally intact rats. Animal care and surgery were performed with the National Institute of Health and American Physiological Society's guidelines for animal safety and care, with protocol approval form the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Rats were either exposed to room air or CIH in Oxycycler chambers (76.2 X 50.8 X 50.8 cm, BioSpherix, Lacona, NY, USA) (8-minute cycles of 5 minutes of 10% O_2 and 3 minutes of 21% O_2) delivered during 1700 to 0500h for 8 days. Rats exposed to CIH protocols had an apnea/hypopnea index (AHI) of 8, indicating mild to moderate sleep apnea. GDX male rats were also implanted with either crystalline testosterone-filled or 2 cholesterol filled continuous release Silastic implants (Steraloids, Newport, RI). This resulted in a 2-3 ng/mL circulating testosterone level that are consistent with average diurnal testosterone levels in rats [16]. Rats were then anesthetized with isoflurane (2-3%) and sacrificed by decapitation [16]. Brains were stored at a temperature of -80°C until ready for dissection.

Rats were separated into six treatment groups: Gonadally Intact Normoxic (n=6), Gonadally Intact Hypoxic (n=4), Gonadectomized Normoxic (n=5), Gonadectomized Hypoxic (n=6), Gonadectomized Normoxic given TRT (n=5), and Gonadectomized Hypoxic given TRT (n=6). Brain tissue was subsequently thawed in 1X phosphate-buffered saline on ice, quickly cut in 2 mm coronal sections using a commercially obtained matrix (Ted Pella, inc., Redding, CA), and then placed on dry ice. Brain tissue was micro-dissected to collect the entorhinal cortex into 1.7 mL microcentrifuge tubes. Samples were stored at -80°C until homogenization.

Homogenization

Tissue samples were homogenized in a RIPA lysis buffer cocktail involving phosphatase inhibitor, dithiothreitol, and EDTA while placed on ice. 70 to 100 microliters of RIPA lysis buffer cocktail were used per sample, depending on the amount of entorhinal cortex collected. Tissue was vortexed and sonicated three times, with the sonicator wiped down three times with ETOH then three times with deionized water. Cell lysate (homogenized tissue samples in RIPA) was then centrifuged at 12,000g for 20 minutes at 4°C, and then the supernatant was collected and transferred to a clean microcentrifuge tube. Protein concentration was quantified via BCA assay, according to the manufacturer's protocol. Following a BCA assay, tissue samples were then boiled to denature contained proteins.

Western Blots

Equal volumes of denatured tissue samples (20 ug) were loaded into BioRad 4-20% polyacrylamide 15-well gels. Samples were then run via electrophoresis at 25 mA in a Tri-glycine

running buffer then transferred into a PVDF membrane at 80 V for 2.5 hours. PVDF membranes were equilibrated using methanol and transfer buffer.

Following the transfer from gel to membrane and a 10-minute wash using TBS-tween (TBST) at room temperature, membranes were blocked in 5% milk TBST solution for 1 hour. Membranes were then be exposed to 10 mL 1% milk TBST with primary antibodies. Primary antibodies used were the following: mouse mouse-Spectrin (EMD Millipore Corporation MAB1622) (1:1000), rabbit anti-GFAP (Abcam ab7260) (1:1000), rabbit anti-COX2 (Abcam ab15191) (1:1000), and mouse anti-beta Actin (GeneTex GTX629630). Following an overnight incubation period at 4˚ Celsius and subsequent wash with TBST, 10 mL of Goat anti-Mouse IgG secondary antibody (Invitrogen REF 31430) (1:5000) or 10 mL of Goat anti-Rabbit IgG secondary antibody (Invitrogen REF 31460) (1:5000) was applied to the membrane. Following a 45-minute incubation in the secondary antibody at room temperature, the membrane was washed again in TBST and protein bands were imaged using West Pico enhanced chemiluminescence detection assay. A Syngene G:Box was used to image the membrane. Following imaging, the membrane was stripped using a western blot stripping buffer (Thermo Scientific REF 21059) and re-probed with other primary antibodies.

Quantitative and Statistical Analysis

Using the NIH Image J software for densitometry, protein concentrations of COX2 and GFAP were normalized to beta-actin protein to determine protein expression. The calpain cleavage bands and caspase-3 cleavage bands of Spectrin were normalized to total Spectrin values. Statistical analysis involved IBM SPSS software, which is presented as mean ± SEM. A two-way

ANOVA was used followed by a Tukey's post hoc test. Statistical significance will be set at $p \leq 0.05$ with trends set to $p < 0.10$.

RESULTS

CIH did not impact protein levels of COX2, GFAP, and Calpain cleavage of Spectrin in the entorhinal cortex of young adult male rats, regardless of hormone status (**Table 1)**. Therefore, CIH and normoxic groups were combined to examine the impact of hormone status on COX2, GFAP, and Calpain cleavage of Spectrin on the entorhinal cortex.

When we collapsed the CIH and normoxic treatment groups together to examine the impact of hormone treatment on oxidative stress and inflammation in the entorhinal cortex, significant differences were observed. Gonadally intact rats had significantly less neuronal inflammation, as measured by COX2, in the entorhinal cortex ($F_{2,28} = 9.764$, $p \leq 0.05$) compared to GDX and TRT treated male rats (**Figure 2 A**). Similarly, we observed a trend of decreased astrocytic inflammation in the entorhinal cortex, as evidenced by decreased GFAP protein, in gonadally intact male rats compared to GDX and TRT treated rats ($F_{2,29} = 3.074$, $p = 0.06$) (**Figure 3 A**). However, no differences in neuronal oxidative stress, as evidenced by Calpain cleavage of Spectrin, were observed in any of the treatment groups ($F_{2,29} = 0.310$, $p > 0.05$) (**Figure 4 A**). Similarly, no differences in neuronal apoptosis, as measured by Caspase-3 cleavage of Spectrin, were observed ($F_{2,29} = 0.230$, $p > 0.05$) (**Figure 5 A**). These results show that TRT does not impact oxidative stress and inflammation in the entorhinal cortex of male rats.

Further analysis of the data showed that there was an unaccounted variable in the experimental design, which was surgical isoflurane. GDX and TRT treated rats were exposed to less than 30 minutes of 2-3% isoflurane during surgery to remove testes and silastic capsule implantation of hormones. Since there was no statistical significance between the GDX and TRT treatment groups on any of the dependent variables measured, the GDX and TRT treated groups

were combined into an “Anesthesia” group with the gonadally intact male rats renamed as “No Anesthesia”.

Exposure to 2-3 % isoflurane significantly increased neuronal inflammation, as measured by COX2, in the entorhinal cortex ($F_{1,29} = 19.118$, $p \leq 0.05$) compared to No Anesthesia treated male rats (**Figure 2 B**). Similarly, we observed increased astrocytic inflammation in the entorhinal cortex, as evidenced by increased GFAP protein, in Anesthesia exposed male rats compared to No Anesthesia treated rats ($F_{1,30} = 6.347$, $p \leq 0.05$) (**Figure 3 B**). However, isoflurane anesthesia did not impact neuronal oxidative stress ($F_{1,30} = .476$, $p > 0.05$) (**Figure 4 B**). Similarly, isoflurane did not affect neuronal apoptosis, as measured by Caspase-3 cleavage of Spectrin, were observed ($F_{1,30} = 0.442$, $p > 0.05$) (**Figure 5 B**). These results show that 2-3% isoflurane during a surgery that lasted less than 30 minutes increased neuronal and astrocytic inflammation in the entorhinal cortex of male rats.

Treatment		COX2 Mean (S.D.)	GFAP Mean (S.D.)	Calpain Mean (S.D.)	Significance
Young Intact	Normoxic	28.78 (19.93)	67.30 (22.87)	169.80 (94.89)	Not significant
	CIH	25.34 (7.87)	48.22 (44.48)	109.03 (35.31)	
GDX	Normoxic	96.87 (58.69)	82.54 (14.96)	143.73 (29.93)	Not significant
	CIH	136.04 (45.09)	102.46 (21.32)	168.30 (45.79)	
GDX + TRT	Normoxic	105.54 (84.42)	101.03 (59.38)	178.47 (103.67)	Not significant
	CIH	98.61 (35.44)	90.06 (46.25)	160.12 (48.76)	

Table 1. CIH had no impact on inflammation or oxidative stress related proteins in the entorhinal cortex, regardless of hormone treatment. (Young intact normoxic = 6, Young intact CIH = 4, GDX normoxic = 5, GDX CIH = 6, GDX + TRT normoxic = 5, GDX + TRT CIH = 6)

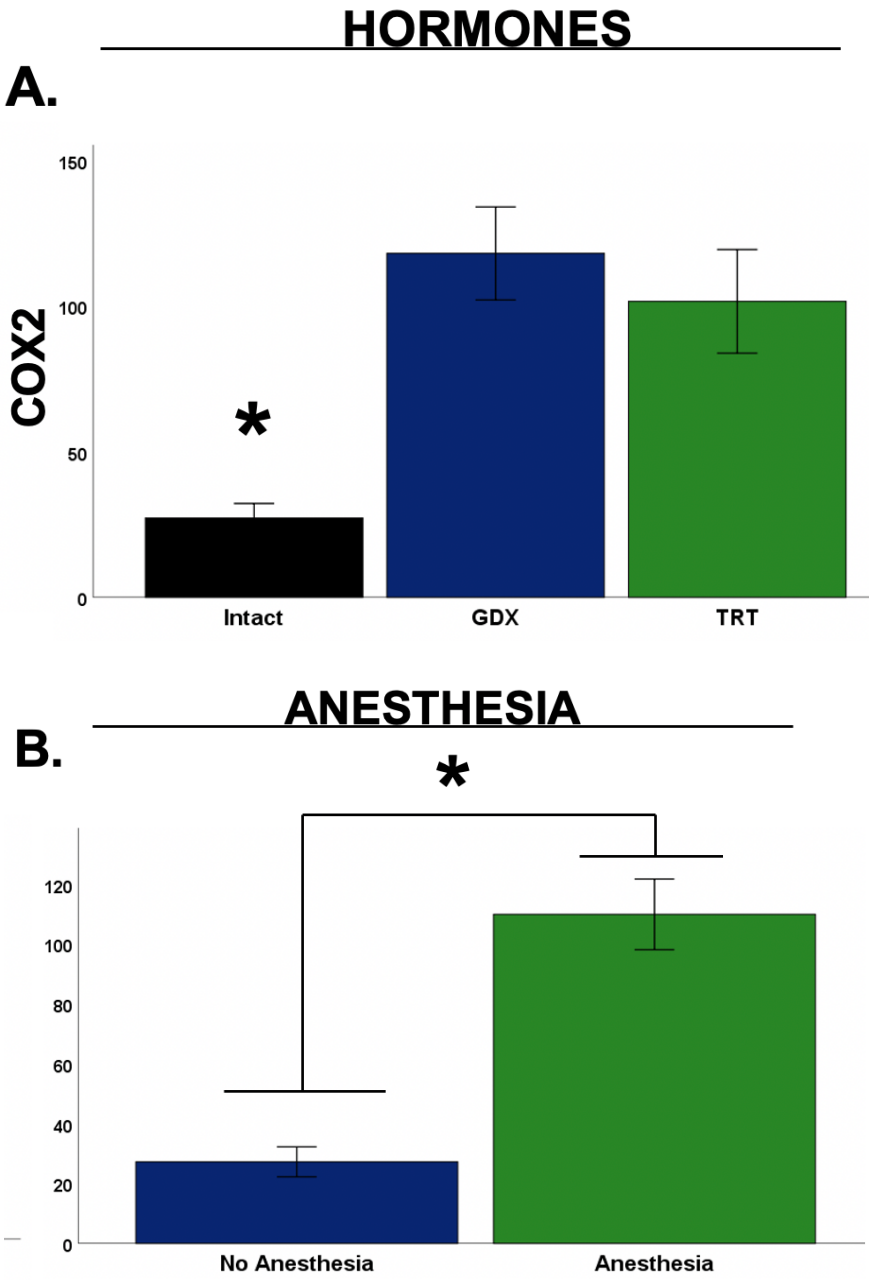

Figure 2. A. Gonadally intact rats had significantly less COX2 protein than the other treatment groups (Intact= 9, GDX= 11, TRT= 11). B. Anesthesia significantly increased COX2 (No Anesthesia= 9, Anesthesia= 22). *= $p \leq 0.05$

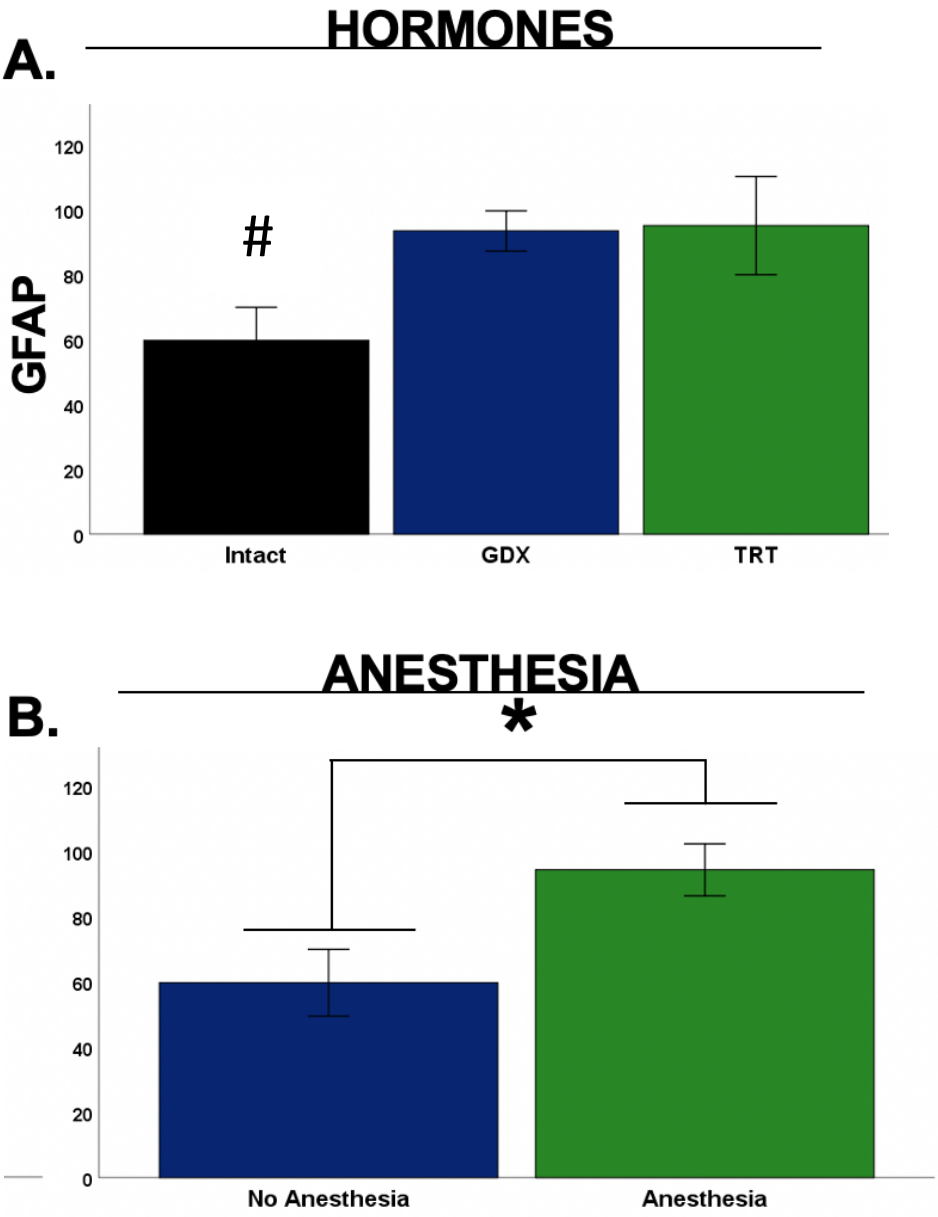

Figure 3. A. Gonadally intact rats showed a trend of less GFAP protein than the other treatment groups (Intact= 10, GDX= 11, TRT= 11). B. Anesthesia significantly increased GFAP (No Anesthesia= 10, Anesthesia= 22). *= $p \leq 0.05$

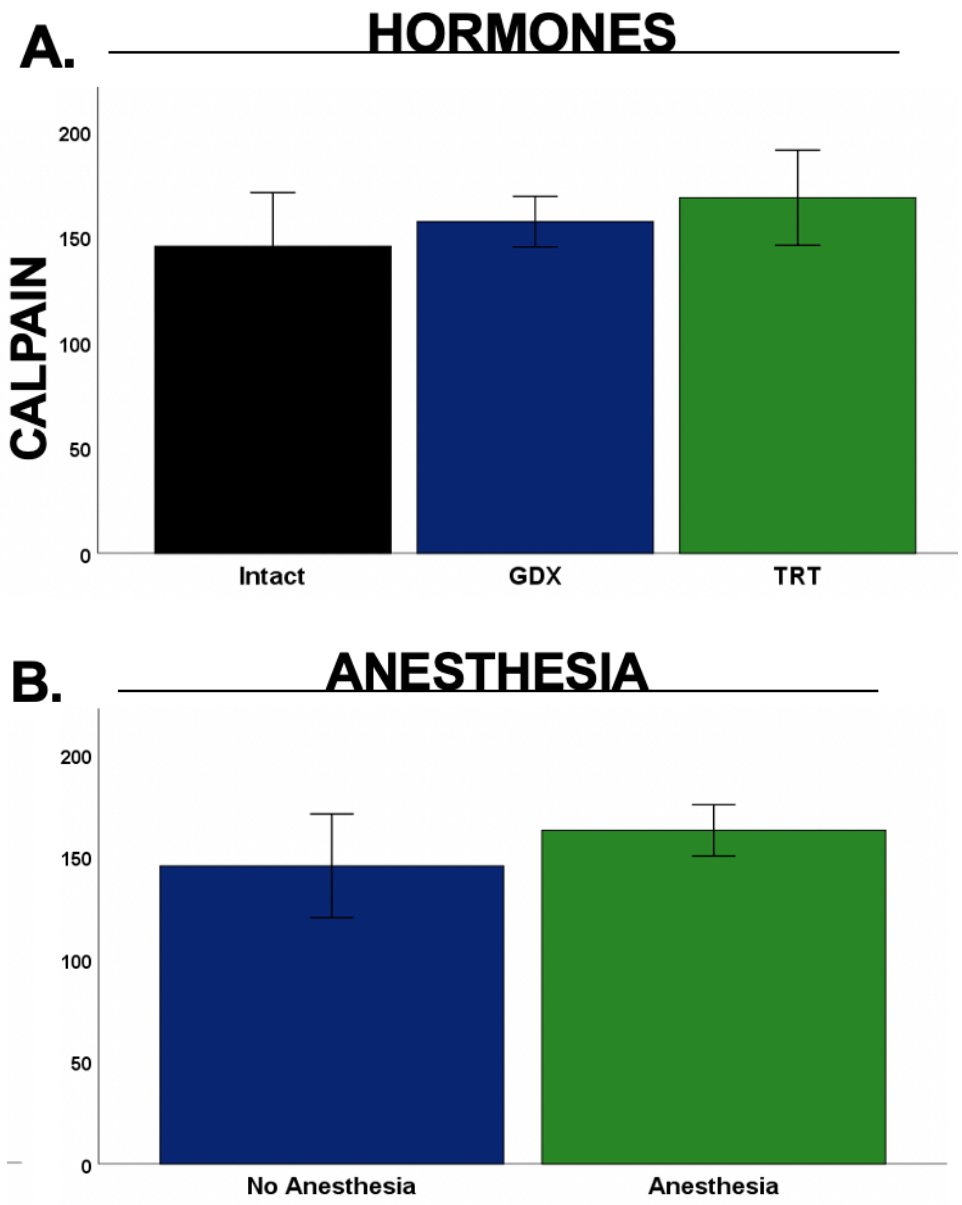

Figure 4. A. Gonadally intact rats did not have significantly less Calpain cleavage of Spectrin protein than the other treatment groups (Intact= 10, GDX= 11, TRT= 11). B. Anesthesia did not significantly increase Calpain cleavage of Spectrin (No Anesthesia= 10, Anesthesia= 22).

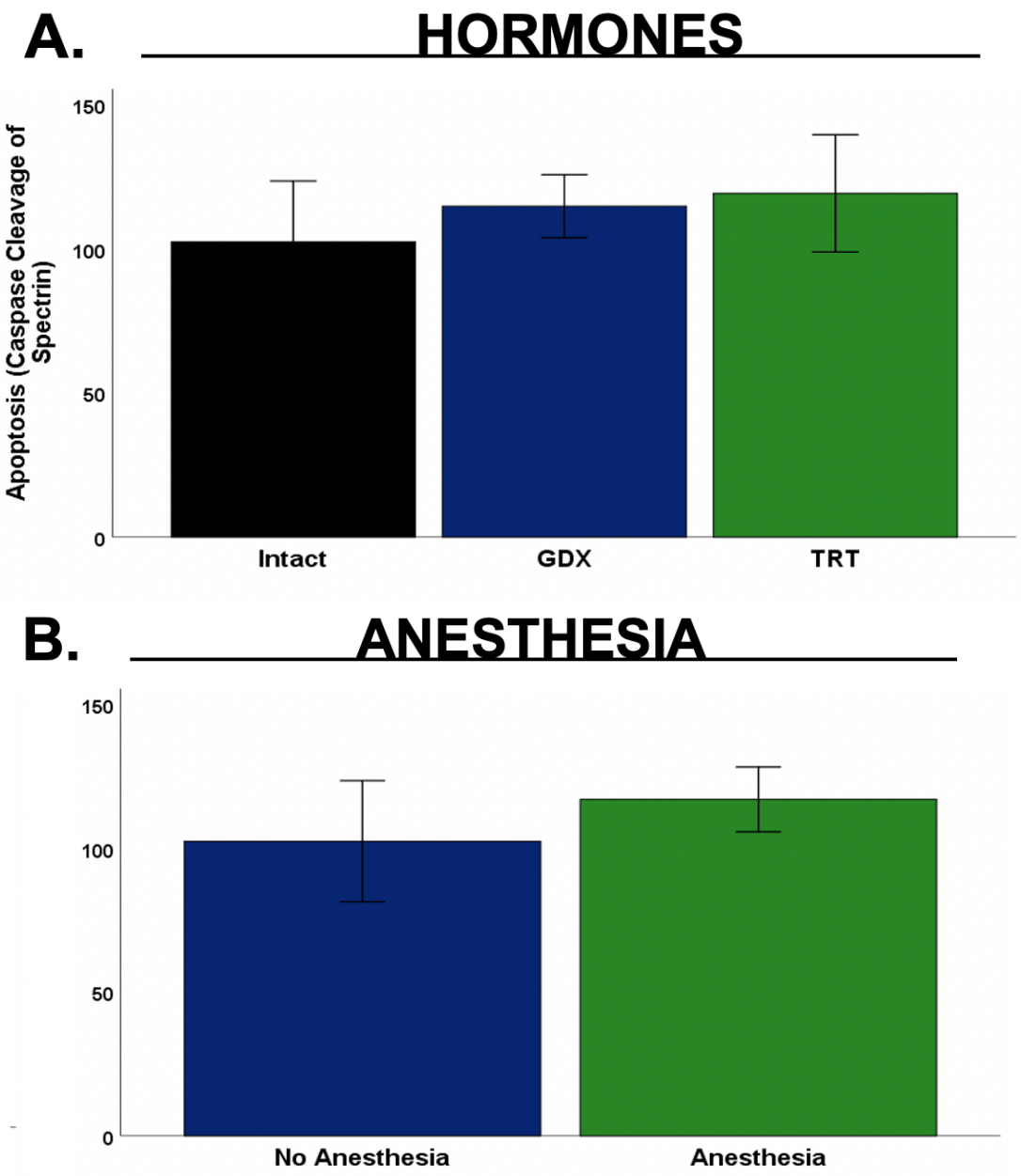

Figure 5. A. Gonadally intact rats did not have significantly less Caspase-3 cleavage of Spectrin protein than the other treatment groups (Intact= 10, GDX= 11, TRT= 11). B. Anesthesia did not significantly increase Caspase-3 cleavage of Spectrin (No Anesthesia= 10, Anesthesia= 22).

DISCUSSION

The hypothesis for this study stated that CIH would increase oxidative stress and inflammation related proteins in the entorhinal cortex of gonadally intact rats, while neither GDX nor TRT would increase expression of oxidative stress or inflammation related markers in the same region. This hypothesis was disproven. Neither CIH nor TRT impacted oxidative stress and inflammation related proteins in the entorhinal cortex of young adult male rats (**Figure 2-5)**. These results are consistent with our prior studies showing a lack of effect of CIH and androgens on oxidative stress and inflammation related proteins in the entorhinal cortex [6].

Although we disproved our original hypothesis, analysis of the data showed an interesting finding. Our treatment groups had an unaccounted independent variable. Gonadally intact rats had not undergone a sham surgery under isoflurane (2-3%), whereas GDX and TRT treated male rats underwent isoflurane surgery for less than 30 minutes. Upon re-analysis of the data by the independent variable of isoflurane exposure, rats that had undergone isoflurane (2-3%) surgery exhibited elevated neuronal and astrocytic inflammation in the entorhinal cortex compared to rats that were never exposed to isoflurane (**Figure 2-3**).

Within rodents, previous studies have shown that isoflurane has similar physiological effects as well as negatively impacting cognitive function. Isoflurane can increase interleukin 1β, an inflammation mediator. This increase in inflammation has been linked to an impairment of learning ability within rodents [29, 30]. Prior studies have shown that low levels of isoflurane (1%) can impair spatial memory in mice [31]. These effects on spatial memory can be exacerbated by repeated isoflurane exposures [32].

Reports of isoflurane causing cognitive impairment have also been noted in humans [33, 34]. Similar to what we observed in our results, in humans isoflurane can increase

neuroinflammation, as evidenced by increased interleukin-6 levels [35]. Furthermore, isoflurane has been shown to increase tau proteins and β-amyloid (Aβ) protein levels within cerebrospinal fluid, leading to an increase in likelihood of Alzheimer's Disease occurrence in more elderly individuals [36-38]. However, the effects of isoflurane on cognitive function in humans directly remains unstudied or inconclusive [39]. Based on our results, short exposure to isoflurane can impact the brain at multiple cellular sites, such as the neurons and the astrocytes.

One form of combating the physiological effects of isoflurane involves the use of acetaminophen, which has been shown to selectively inhibit COX2 production [40, 41]. By decreasing inflammation levels, acetaminophen can decrease COX2 expression, and through that decease levels of inflammation within the entorhinal cortex. More research can be done in this field to determine the results of potential interactions between isoflurane and acetaminophen.

This study does have some limitations. One of the major limitations is that cognitive behaviors were not assessed in the rats. Therefore, we are unable to associate the cellular oxidative stress and inflammation outcomes in the entorhinal cortex with functional cognitive data. In addition, within the original study older rats did not undergo isoflurane associated surgery, and thus we are unable to address the impact of isoflurane on brain inflammation in an aged model.

FUTURE DIRECTIONS AND CONCLUSIONS

When looking at future work that can be pursued in this field, potential variables could be explored. First, length of exposure to isoflurane dosage could be varied to understand how a short-term exposure compared to long-term exposure could affect inflammation and oxidative stress levels within the rats. Also, as seen above, dose of exposure could affect levels of impact on cognitive function when looking at spatial memory testing. Age of rats at time of exposure can also be tested, in order to see how young rats compare to older rats, who may have less naturally produced testosterone and decreased neuroprotection to the effects of isoflurane.

In conclusion, isoflurane (2-3%) significantly increased levels of COX2 and GFAP, markers of inflammation, within the entorhinal cortex of young rats. However, oxidative stress in the entorhinal cortex was unaffected by short term exposure to isoflurane (2-3%) exposure.

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